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SHORT COMMUNICATION

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Topographic changes in nascent and early mesoderm in amphioxus embryos studied by DiI labeling and by in situ hybridization for a *Brachyury* gene

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Abstract In amphioxus embryos, the nascent and early mesoderm (including chorda-mesoderm) was visualized by expression of a *Brachyury* gene (*AmBra-2*). A band of mesoderm is first detected encircling the earliest (vegetal plate stage) gastrula sub-equatorially. Soon thereafter, the vegetal plate invaginates, resulting in a cap-shaped gastrula with the mesoderm localized at the blastoporal lip and completely encircling the blastopore. As the gastrula stage progresses, DiI (a vital dye) labeling demonstrates that the entire mesoderm is internalized by a slight involution of the epiblast into the hypoblast all around the perimeter of the blastopore. Subsequently, during the early neurula stage, the internalized mesoderm undergoes anterior extension mid-dorsally (as notochord) and dorsolaterally (in paraxial regions where segments will later form). By the late neurula stage, *AmBra-2* is no longer transcribed throughout the mesoderm as a whole; instead, expression is detectable only in the posterior mesoderm and in the notochord, but not in paraxial mesoderm where definitive somites have formed.

Key words Mesoderm · Notochord · *Brachyury* · Amphioxus

Introduction

Amphioxus (phylum Chordata: subphylum Cephalochordata) is widely thought to be the closest living invertebrate relative of the vertebrates. Because of its exceptional phylogenetic importance, amphioxus has been intensively studied by biologists in general and by embryologists in particular. The early embryology of amphioxus has often been described and may be summarized briefly as follows: The fertilized egg (less than 150 μm in diameter and containing only a modest amount of yolk) undergoes cleavage to form the blastula, which is a hollow ball of cells surrounding a central blastocoel. Subsequently, a flattening of one side of the blastula (the vegetal plate) signals the start of the gastrula stage. As gastrulation progresses, the vegetal plate invaginates, largely obliterating the blastocoel and creating a new space, the archenteron, which opens to the exterior via a blastopore. The lip of the blastopore is where the outer cell layer (epiblast) meets the inner cell layer (hypoblast). One side of the blastoporal lip is dorsal – as subsequent development demonstrates.

Although the general features of amphioxus gastrulation are not controversial, some of the details remain in dispute. For instance, claims that the dorsal lip can be distinguished from the rest of the blastoporal lip on the basis of cell size or overall shape of the blastopore (reviewed by Conklin 1932) have not been substantiated by more recent work (Hirakow and Kajita 1991). It is also not clear where the early boundaries separating the three primary germ layers should be drawn during the gastrula stage after the advent of the mesoderm. Although the whole hypoblast can be referred to as mesendoderm, it is preferable to divide this tissue into a region of endoderm and a region of mesoderm. For the present study, we took advantage of the recent finding that the expression domains of *Brachyury* genes reliably mark forming mesoderm in vertebrates (De Robertis et al. 1994) as well as in amphioxus (Holland et al. 1995; Terazawa and Satoh 1995). We gave special attention to the earliest gastrula stage (not included in previous studies of amphioxus *Brachyury*) and to the anterior expansion of the mesodermal zone during the early neurula stage.

Another controversy about amphioxus gastrulation concerns whether epiblast cells are involuted around the blastoporal lip to become hypoblast. Sobotta (1897), MacBride (1898, 1909) and Morgan and Hazen (1900) thought there was no involution at all. In contrast, Lwoff (1892), Cerfontaine (1906) and Conklin (1932) believed

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involution takes place all around the blastoporal lip and is *especially pronounced* dorsally. Finally, Klaatsch (1897) proposed involution occurs all around the blastoporal lip *except* dorsally. Therefore, to determine which, if any, of these versions of amphioxus gastrulation is correct, we labeled epiblast cells with a vital dye (DiI) and followed their subsequent movements.

Materials and methods

Ripe males and females of the Florida amphioxus, *Branchiostoma floridae*, were collected in Tampa Bay, Florida. The animals were spawned in the laboratory and the embryos raised at 23.0 ± 0.5°C. We used *Brachyury* expression as a marker for mesoderm. *Branchiostoma floridae* has two *Brachyury* genes, *AmBra-1* and *AmBra-2* (Holland et al. 1995). An antisense riboprobe comprising about 850 base pairs starting from the 3′ end of *AmBra-2* was synthesized and whole mount in situ hybridizations of *B. floridae* embryos were prepared according to the methods in Holland et al. (1996). Our in situ patterns with *AmBra-2* were indistinguishable from in situ patterns with *AmBra-1* illustrated in Holland et al. (1995).

For DiI labeling, fine forceps were used to remove the fertilization envelope from blastulae (4 h), which fortuitously adhered to the bottom of 3.5-cm plastic petri dishes while continuing to develop normally. At 5.5 h, cap-shaped gastrulae (Figs. 1A, 2B) with the blastopore pointing approximately upward were selected for labeling. A glass capillary tube was filled with Fast DiI oil (D-3899; Molecular Probes, Eugene, Ore.) and then immersed in sea water, causing the dye to crystallize on the tube tip. To make each spot, the crystallized dye was applied with a micromanipulator by gently touching the surface of the gastrula for about 2 min. For each of ten embryos, the epiblast was dyed with eight spots (each about 30 μm in diameter) arranged radially around the blastopore. An unstained zone of epiblast of about 20 μm remained between each spot and the blastoporal lip; the position of a single spot is shown diagrammatically by the hatching in Fig. 1A. An additional 75 cap-shaped gastrulae were dyed with a single spot of DiI each at various positions on the epiblast or hypoblast; 10 of these embryos were stained with a single spot right on the blastoporal lip; the position is shown diagrammatically by the stippling in Fig. 1A.

Dyed embryos were photographed alive under a fluorescence microscope fitted with a DAPI (4,6-diamidino-2-phenylindole) filter. Each embryo was photographed in the same orientation at about 6, 7, and 8 h after fertilization. Soon after, the embryos, by then very late gastrulae, developed cilia, detached from the bottom of the dish, and began to swim. Between 8.5 and 36 h, the swimming embryos were killed in 4% paraformaldehyde for a final observation by fluorescence microscopy.

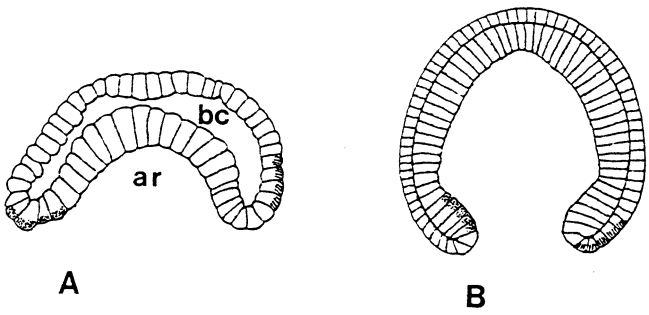

Fig. 1A, B Diagrams of amphioxus gastrulae oriented with the animal pole *upward* and the blastopore side *downward*. **A** Cap-shaped gastrula (6 h after fertilization) with invaginating archenteron (*ar*) and remnant of blastocoel (*bc*); *stippling* indicates the position of a DiI (a vital dye) spot directly on the blastoporal lip and *hatching* indicates the position of a DiI spot 20 μm outside of the blastoporal lip. **B** Very late gastrula (8.5 h after fertilization); the DiI spot originally on the blastoporal lip has been completely involuted (*stippling*) and the DiI spot originally 20 μm outside the blastoporal lip has moved right to the edge of the blastopore (*hatching*)

Results and discussion

No *AmBra-2* expression could be demonstrated by in situ hybridizations of cleavage stages and blastulae of *Branchiostoma floridae*. The first detectable transcripts (Fig. 2A) appeared at the earliest gastrula stage (4.5 h) in a zone of cells encircling the embryo between the animal hemisphere (ectoderm) and the flattened vegetal plate (endoderm). We assume that the cells expressing *AmBra-2* comprise the mesoderm (a term that we will use to include both the mesoderm sensu stricto plus, on the dorsal side, the chorda-mesoderm). The slightly sub-equatorial location of the mesoderm in the early gastrula agrees closely with the position of the presumptive mesoderm revealed by the fate map constructed by Tung et al. (1962) for an earlier (32-cell) stage of amphioxus development.

By the stage of the cap-shaped gastrula (6 h), the vegetal plate has invaginated and *AmBra-2* is expressed strongly all around the lip of the blastopore, partly in epiblast cells and partly in hypoblast cells (Fig. 2B). Soon after, by the stage of the cup-shaped gastrula (7 h), the strongly expressing cells, presumably representing the mesoderm, occur in a broad band located entirely within the lip of the blastopore (Fig. 2C). Thus, the hypoblast is divisible into an endodermal (animal) zone and a mesodermal (vegetal) zone, while the entire epiblast is ectodermal.

Conceivably, the movement of the entire mesodermal zone into the hypoblast might have resulted from initiation of *AmBra-2* expression in additional cells at the hypoblastic edge of the zone accompanied by down-regulation of the same gene at the epiblastic edge of the zone. However, the DiI movements during gastrulation show that this is not a major cause of the internalization of the mesoderm. Instead, movement of the dye spots between 6 and 7 h demonstrates that some of the epiblast is involuted (Fig. 2I,J), probably as a coherent sheet of cells, over the blastoporal lip to become hypoblast. The amount of involution, which is of approximately equal extent everywhere around the blastoporal lip, appears to be sufficient to account for the internalization of the mesoderm.

In comparison to gastrulation in lower vertebrates, gastrulation in amphioxus involves neither exaggerated involution in the region of the dorsal lip of the blastopore nor any marked tendency of epiblast cells to converge toward the dorsal midline. For amphioxus, nothing definite is known about the mechanical factors driving involution (e.g., cell shape and motility in the region of the blastoporal lip). Changes in cell cycle lengths and mitotic patterns might also play a part in tissue level movements

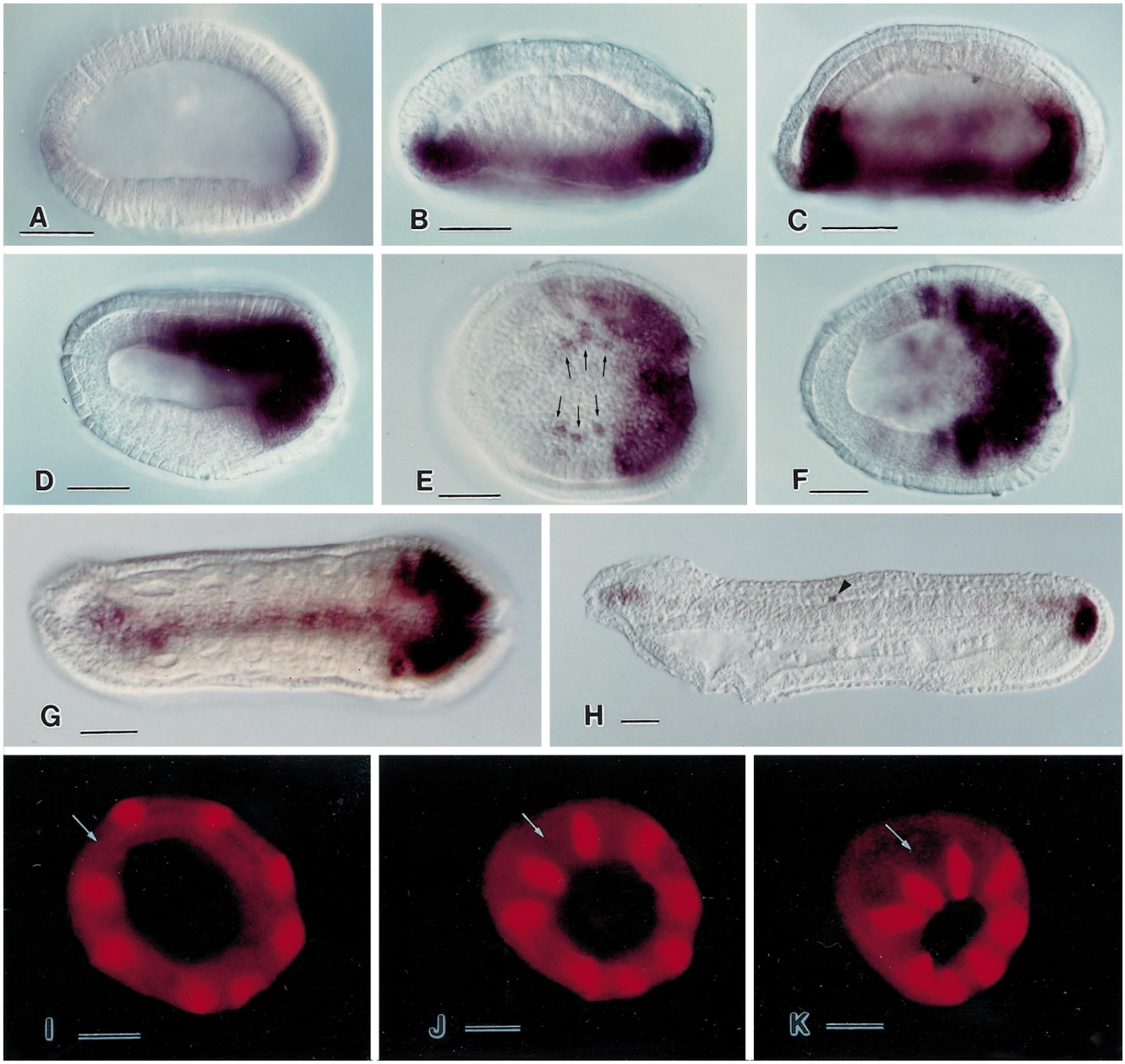

Fig. 2A–K Whole mounts of amphioxus embryos (*all scale lines* 50 μm). **A–H** In situ preparations showing expression of *AmBra-2*; **A–C** are oriented with the animal pole *upward*; **D–H** are oriented with posterior (approximately equivalent to vegetal) pole toward *right*. **A** Side view of early gastrula (4.5 h). **B** Side view of cap-shaped gastrula (6 h). **C** Side view of cup-shaped gastrula (7 h). **D** Side view of early neurula (9.5 h); dorsal surface is *up*. **E** Dorsal view of early neurula (9.5 h); dorsal wall of hypoblast in focus; *arrows* indicate small clusters of cells expressing *AmBra-2*. **F** Dorsal view of early neurula (9.5 h) with focal plane deeper than in **E**. **G** Dorsal view of late neurula (16 h); *AmBra-2* expressed strongly in posterior mesoderm and moderately along notochord; no detectable expression in the definitive segments on either side of midline. **H** Side view of 24-h embryo; dorsal surface is *up*; *AmBra-2* expressed strongly in posterior mesoderm, but weakly in notochord except at anterior and posterior ends; black pigment cell in dorsal nerve cord indicated by *arrowhead*. **I–K** Living gastrula; the *arrow* indicates the dorsal side (as determined from bright field microscopy); approximately vegetal pole views of single specimen marked with eight spots of DiI and photographed by fluorescence microscopy at 6 h (**I**), 7 h (**J**), and 8 h (**K**)

during gastrulation, but no cell kinetic data are yet available.

During the last part of gastrulation, there is little further involution (Figs. 1B, 2K). At later developmental stages, after the blastopore has closed, the dye spots remain in the ectoderm – on the outside of the embryos and larvae, except dorsally where they occur in the ectodermally derived dorsal nerve cord. Our observations on the fate of single spots of DiI applied at various positions to the epiblast and hypoblast of gastrulae (data not shown) agreed well with the earlier results of Tung et al. (1962), who stained amphioxus blastomeres with Nile blue sulfate and followed their fates. In 20 embryos, cells dyed with DiI right on the blastoporal lip at the stage of the cup-shaped gastrula were internalized during the next few hours (Fig. 1B, stippling), and at least some of them were subsequently recruited into mesodermal somites.

Changes in *AmBra-2* expression domains after the end of gastrulation are shown in Fig. 2D–H; in these in situ diagrams, the blastopore (or approximate posterior end) – which is toward the bottom of Fig. 2A–C – is oriented toward the right. In the early neurula (9.5 h), the internalized mesoderm, as marked by *AmBra-2* expression, still occupies the most posterior (formerly animal) region of the hypoblast and has also begun to spread anteriorly along the dorsal and dorsolateral walls of the hypoblast (Fig. 2D). This spread could result from some combination of: (1) changes in individual cell shape or packing, (2) migration of expressing cells individually into non expressing areas, or (3) induction of new expression in previously non-expressing areas. One of the last two causes is probably responsible for the presence of isolated clusters (three to five cells each) of expressing cells (indicated by arrows in Fig. 2E) in the dorsal roof of the hypoblast. The banded expression in the dorso-lateral wall of the hypoblast (Fig. 2F) has previously been clearly described by Terazawa and Satoh (1995). Each band is presumably localized where a somite will later form.

By the later (16 h) neurula (Fig. 2G), *AmBra-2* expression is no longer detectable throughout the mesoderm. Expression remains strong in the posterior hypoblast (which can now be referred to as posterior or peristomial mesoderm) and, just anterior to that, in a separate dorsolateral stripe where the newest somite will soon form. In contrast, there are no detectable transcripts of the gene in the paraxial mesoderm where the definitive somites have formed.

By the late neurula stage, there is also moderate expression of *AmBra-2* in cells of the notochord (as previously demonstrated by Holland et al. 1995). The striking anterior extension of notochordal expression presumably results from migration and intercalary movements of cells already expressing the gene and/or the induction of transcription in previously non-expressing cells.

Following the neurula stage, amphioxus *Brachyury* expression has already been described by Holland et al. (1995), and we confirmed their findings. Briefly, in the 24-h embryo (Fig. 2H), the cells of the posterior mesoderm still contain abundant transcripts of *AmBra-2*, although there are no longer any strong expression bands just anterior to that zone; moreover, expression continues in the notochord, but is weakening, except near its anterior and posterior ends where new notochordal cells are presumably differentiating. By 60 h after fertilization, in situ hybridization no longer demonstrates *AmBra-2* expression in any of the tissues of the amphioxus larva.

During gastrulation of both amphioxus and vertebrates, the expression patterns of *Brachyury* homologs are expressed first in internalizing mesoderm. Later, *AmBra-2* in amphioxus is expressed throughout the newly internalized mesoderm and in the forming notochord; in vertebrates, by contrast, *Brachyury* expression is rapidly down-regulated in newly internalized mesoderm except in the tailbud and notochord (De Robertis et al. 1994). It is more difficult to relate expression patterns of *Brachyury* homologs in amphioxus and other invertebrates. For instance, the *Brachyury* homolog in *Drosophila* embryos is expressed in the hindgut epithelium (Kispert et al. 1994), but it would be premature to suggest an homology between the insect hindgut and chordate notochord. There is an obvious need for description of *Brachyury* genes and their developmental expression in a wide spectrum of animal phyla.

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